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Genetic variation for big-vein symptom expression and resistance to *Mirafiori lettuce big-vein virus* in *Lactuca virosa* L., a wild relative of cultivated lettuce

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Abstract *Lactuca virosa* L. is a wild relative of lettuce that is potentially an important source of resistance to big-vein disease, an economically damaging disease of lettuce. Identification of *L. virosa* accessions with resistance to *Mirafiori lettuce big-vein virus* (MLBVV), the disease causing agent, may be useful for lettuce breeding. The objectives of this research were to determine the genetic variation for big-vein symptom expression and MLBVV accumulation in diverse *L. virosa* accessions. Greenhouse testing was conducted to characterize variation for symptom expression 90–100 days after planting (DAP) with 70 *L. virosa* accessions in unreplicated experiments in 2001 and 2003, and with 10 accessions in an experiment with 3 replications conducted in 2004. In 2005, six replications of seven accessions were evaluated for the percentage of symptomatic plants 120 DAP and 180 DAP in a growth chamber experiment. Reverse transcription-polymerase chain reaction or nucleic acid spot hybridization was used to determine MLBVV presence or absence at each reading date. Genetic variation for symptom expression was confirmed among the *L. virosa* accessions, although the majority of tested accessions did not express big-vein symptoms. Symptomless infections were discovered, although accumulation of MLBVV to detectable levels appeared to be a slow process in *L. virosa*. Genetic variation for the incidence of MLBVV positive plants was identified within symptomless accessions, and suggests that symptom expression and MLBVV resistance may be independent factors contributing to big-vein resistance. Regardless, symptomless accessions with low MLBVV incidence were identified, and should be useful for breeding new big-vein resistant cultivars.

Keywords *Lactuca sativa* L. · Breeding · *Compositae* · Disease resistance · Virus resistance · *Ophiovirus* · MLBVV

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Introduction

Lactuca virosa L. is an evolutionarily divergent wild relative of *L. sativa* with a geographic distribution centered around the Mediterranean basin (Koopman et al. 1998; Koopman et al. 2001; Lebeda et al. 2004). *Lactuca virosa* is an important source of genetic resistance to numerous viral, fungal, bacterial, and insect pests of lettuce (Lebeda et al. 2007). *Lactuca virosa* could be an important source of complete resistance or potential immunity to lettuce big-vein disease (Hayes et al. 2006). Big-vein is an economically damaging disease complex of lettuce (*Lactuca sativa* L.) that occurs in lettuce production regions around the world (Colariccio et al. 2003; Fujii et al. 2003; Jagger

and Chandler 1934; Latham and Jones 2004; Lot et al. 2002; Roggero et al. 2000; Rosales et al. 2004). The disease causing agent is *Mirafiori lettuce big-vein virus* (MLBVV), which is vectored by the soil-borne fungus *Olpidium brassicae* (Lot et al. 2002; Roggero et al. 2000). While genetic resistance from *L. virosa* offers an effective and economically feasible method of controlling big-vein, only partial resistance exists in cultivated lettuce (Bos and Huijberts 1990; Fujii et al. 2003; Latham and Jones 2004; Ryder and Robinson 1995).

Complete resistance to big-vein, the consistent and complete absence of symptoms, was described in the *L. virosa* accessions IVT278 and IVT280 (Bos and Huijberts 1990; Hayes et al. 2006). Campbell (1965) reported weak symptom expression in *L. virosa*, although the accession name was not indicated. Efforts to introgress resistance from *L. virosa* into lettuce using accession IVT280 have been reported (Hayes and Ryder 2007). Hybrid breeding populations did not contain individuals with complete resistance, but did have variation for partial resistance that was likely based on novel alleles derived from *L. virosa*. The failure of this effort may be related to the difficulty of breeding with *L. virosa*, which requires the use of bridge crosses with *L. serriola*, colchicine doubling, or embryo rescue (Eenink et al. 1982; Maisonneuve et al. 1995; Thompson and Ryder 1961). More effort is needed to develop lettuce cultivars with complete big-vein resistance derived from *L. virosa*. It is not clear whether the inability to introgress resistance from IVT280 into *L. sativa* to date is due to a specific problem with accession IVT280. Additional accessions with complete resistance to big-vein are needed to answer this question. Furthermore, susceptible *L. virosa* accessions would be useful for determining the biology and genetics of big-vein resistance in *L. virosa*. The objective of this research was to determine the genetic variation for big-vein symptom expression and MLBVV accumulation in *L. virosa*.

Materials and methods

Greenhouse and growth chamber testing of big-vein resistance

All greenhouse or growth chamber experiments were performed according to Ryder and Robinson (1995). Inoculum was produced in the greenhouse by growing big-vein symptomatic plants in 15 cm pots containing *O. brassicae* infested field soil collected from the USDA-ARS research station in Salinas, CA. The field soil used to grow symptomatic plants was collected from the same location for each experiment. *Mirafiori lettuce big-vein virus* (MLBVV) isolates obtained from these plants were closely related to other MLBVV isolates in California, Arizona, Europe, and Japan (Hayes et al. 2006). At the time of inoculation, a suspension of greater than 30,000 *O. brassicae* zoospores per ml was prepared from 6 symptomatic plants by macerating the roots in water. Seedlings were germinated in a 2:1 (sand:field soil) potting mix and grown for three weeks. Inoculations were conducted by watering these seedlings with the zoospore suspension on two occasions separated by 48 h. Each seedling received approximately 5.2×10^5 zoospores. Plants were grown at 18°C and symptoms evaluated over an eight week period, approximately 90–100 days after planting (DAP) unless otherwise stated.

Mirafiori lettuce big-vein virus detection

Tissue samples were ground in liquid nitrogen, total nucleic acid was extracted according to the method of Dellaporta et al. (1983), and extracts were stored at −80°C. Primers CP829F and CP1418R were used for amplification of MLBVV by RT-PCR as described previously (Hayes et al. 2006). Positive and negative controls consisted of MLBVV infected *L. sativa* and *L. virosa*, and greenhouse grown *L. virosa* and *L. sativa* not exposed to MLBVV, respectively. RT-PCR amplicons were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide to determine the presence or absence of target bands. Nucleic acid spot hybridization was performed using a probe made against the coat protein gene of MLBVV as described previously (Hayes et al. 2006).

2001 and 2003 Unreplicated greenhouse experiments

Unreplicated plots of *L. virosa* accessions and *L. sativa* control cultivars were evaluated in 2001 (11 accessions) and 2003 (63 accessions) (Table 1). Up to 12 plants per accession were evaluated, and notes on putative symptoms were recorded. In cases where plants had vein banding typical of big-vein disease,

Table 1 Big-vein reaction in an unreplicated greenhouse screen of 70 *L. virosa* accessions and the *L. sativa* cultivars Great Lakes 65 and Pavane

Salinas accession number	Original accession number	2001 Reaction	2003 Reaction
IVT278	NA	NT	None
IVT280	NA	NT	None
SAL010	Acc. 3350	None	NT
SAL012	B-1	None	NT
SAL013	B-2	None	NT
SAL014	B-3	Leaf Crinkling	Leaf Crinkling
SAL015	B-4	None	NT
SAL020	France 3	None	NT
SAL021	France 3-1	None	NT
SAL024	France 6	None	NT
SAL031	Japan 5	NT	None
SAL093	Unknown	NT	None
SAL094	Lactuca virosa 34	NT	Leaf Crinkling
SAL095	Lactuca virosa 89	NT	None
SAL096	Unknown	NT	None
SAL097	Unknown	NT	None
SAL098	Unknown	NT	None
SAL099	Santa Cruz, CA	NT	None
SAL107	CGN04678	None	None
SAL108	CGN04679	None	None
SAL109	CGN04680	None	None
SAL110	CGN04681	NT	None
SAL113	CGN04950	NT	None
SAL114	CGN04954	NT	None
SAL115	CGN04955	NT	None
SAL116	CGN04956	NT	None
SAL117	CGN04963	NT	None
SAL118	CGN04964	NT	None
SAL119	CGN04970	NT	None
SAL120	CGN04972	NT	None
SAL121	CGN05020	NT	None
SAL122	CGN05077	NT	None
SAL124	CGN05145	NT	None
SAL126	CGN05148	NT	None
SAL129	CGN05266	NT	None
SAL131	CGN05268	NT	None
SAL132	CGN05270	NT	None
SAL135	CGN05283	NT	None
SAL160	CGN05331	NT	None
SAL162	CGN05332	NT	None
SAL163	CGN05333	NT	None
SAL164	CGN05793	NT	None
SAL165	CGN05794	NT	None
SAL167	CGN05816	NT	None

Table 1 continued

Salinas accession number	Original accession number	2001 Reaction	2003 Reaction
SAL168	CGN05869	NT	None
SAL173	CGN05941	NT	None
SAL175	CGN05978	NT	None
SAL177	IVT1398	NT	Vein Clearing (16%)[a]
SAL179	IVT803314	NT	None
SAL180	IVT812222	NT	None
SAL181	IVT812224	NT	None
SAL182	IVT812226	NT	None
SAL183	IVT812230	NT	None
SAL184	IVT831582	NT	None
SAL185	IVT831584	NT	None
SAL186	IVT831586	NT	None
SAL187	IVT831588	NT	None
SAL188	IVT803298	NT	None
SAL193	NPI4772	NT	Leaf Crinkling
SAL194	NPI4772-1	NT	Leaf Crinkling
SAL195	NPI4772-2	NT	Leaf Crinkling
SAL196	NPI4772-3	NT	Leaf Crinkling
SAL197	NPI4772-4	NT	Leaf Crinkling
SAL207	NPI87-47	NT	None
SAL208	NPI87-49	NT	None
SAL209	NPI87-49-1	NT	None
PI274378D	PI274378	NT	None
PI274378B	PI274378	NT	None
PI274375	NA	NT	None
PI271938	NA	NT	None
Pavane	NA	NT	Vein Clearing (42%)
Great Lakes 65	NA	Vein Clearing (100%)	Vein Clearing (92%)

[a] Percentage of plants with vein clearing symptoms

NA, Not applicable; NT, not tested

the percentage of symptomatic plants by the end of the experiment was recorded. Tissue samples were collected from plants with atypical symptoms (stunting, necrosis and leaf curling) to test for the presence or absence of MLBVV using RT-PCR.

2004 Replicated greenhouse experiment

Big-vein resistance was evaluated in a greenhouse experiment with 3 replications of 12 plants. The materials evaluated included *L. virosa* accessions PI271938, SAL012, SAL177, IVT280, and the *L. sativa* cultivars Pavane and Great Lakes 65. In addition, accessions CGN16272, CGN16273, CGN16274, CGN16275, CGN16276, and CGN16277 that were not previously tested were included. Accession CGN16273 was reported to express big-vein symptoms (Johan Schut, Rijk Zwaan, personal communication). The percentage of symptomatic plants at the end of the experiment was calculated, and chi-square goodness-of-fit was used to test whether *L. virosa* accessions are different from the percentage of big-vein symptomatic plants. Tissue was collected from randomly selected plants of CGN16272, CGN16273, CGN16274, IVT280, SAL012, SAL177, and from 6 symptomatic Great Lakes 65 plants at the end of the experiment to determine MLBVV presence or absence using nucleic acid spot hybridization (NASH) (Hayes et al. 2006). This NASH experiment resulted in samples that were clearly positive for MLBVV, samples that were clearly negative for

MLBVV, as well as samples that exhibited weak or faint signals. Retesting the last group using the same technique did not provide a definitive answer. Since the NASH technique is typically quite repeatable, it seemed unwise to categorize these samples as either negative or positive for MLBVV. Therefore, these experiments were analyzed by first calculating the percentage of plants with clearly positive results, and second by calculating the percentage of plants with clearly positive result plus plants with faint NASH results. A chi-square goodness-of-fit test was attempted with the NASH data. While the results indicated a significant difference, many cells of the chi-square contingency table had expected values lower than 1. Consequently, the test was not reported due to the likely unreliability of this χ^2-test.

2005 Replicated growth chamber experiment

A growth chamber experiment to evaluate big-vein resistance was conducted with up to 6 replications of 10 plants per plot using accessions CGN16275, CGN16276, CGN16277, IVT280, PI274378, SAL012, and SAL195. The plants were grown at 18°C for 12 h day lengths. In these experiments, only symptoms that were typical of big-vein were recorded for each plot and the proportion of symptomatic plants was determined at two reading dates, 120 DAP and 180 DAP. Tissue was collected from each plant at both assessment dates to determine MLBVV presence or absence using nucleic acid spot hybridization (NASH), and the proportion of MLBVV positive plants was calculated. All proportion data were transformed to arcsine values, analyzed in Proc Mixed in SAS (Cary, NC) as a randomized complete block design with accession as a fixed effect and block as a random effect. Simultaneous confidence intervals (95%) using the Tukey adjustment for multiple comparisons were calculated to compare treatment means. The data were reported as the percentage of symptomatic plants and the percentage of MLBVV positive plants.

Results

Two years of unreplicated greenhouse experiments identified 62 asymptomatic accessions of *Lactuca virosa*, and one accession, SAL177, with 16% of plants with typical vein banding symptoms (Table 1). The susceptible control Great Lakes 65 and the partially resistant cultivar Pavane also exhibited typical vein banding symptoms. Atypical growth habits or putative symptoms were also observed. Leaf crinkling, epinasty, and necrosis were observed on SAL014 in 2001 and 2003, and in SAL094, SAL193, SAL194, SAL195, SAL196, and SAL197 in 2003 (Table 1). Symptomatic leaf samples were taken from 21 plants of these accessions grown in the 2003 experiment to determine the presence of MLBVV using NASH and RT-PCR. MLBVV was only detected in a single plant from SAL195, and was not detected in any of the other lines exhibiting atypical symptoms. This suggests that MLBVV was not the cause of these symptoms.

Genetic variation for vein banding symptoms typical of big-vein disease was identified among ten accessions of *L. virosa* and Pavane in a greenhouse experiment (Table 2). The percentage of symptomatic plants ranged from 0% (IVT280, PI271938, and SAL012) to 17% (CGN16273), and the difference between accessions was significant (χ^2, 10 df = 19.5; $P < 0.05$). Seven *L. virosa* accessions had at least one symptomatic plant. Symptoms were observed on 9% of plants of the cultivar Pavane, a *L. sativa* cultivar with partial resistance to big-vein. The susceptible cultivar, Great Lakes 65, which was not included in the chi-square analysis, had 89% symptomatic plants. Randomly selected plants from six accessions, in addition to six symptomatic plants from Great Lakes 65, were sampled and tested for MLBVV using NASH. *L. virosa* accessions ranged from 0% (SAL012) to 100% (CGN16274) of plants that were positive for MLBVV (Table 2). Great Lakes 65 had 100% of tested plants positive for MLBVV. Accessions IVT280, SAL177, and SAL012 had varying numbers of samples that resulted in faint spots in the NASH tests, and could not conclusively be determined as MLBVV positive in subsequent retesting. However, if these are also considered to be positive results, it increases the number of MLBVV positive plants to 92% in SAL177, 75% in IVT280, and 17% in SAL012.

Seven *L. virosa* accessions were further tested in a growth chamber experiment for big-vein symptom expression, and genetic variation for the percentage of symptomatic plants was identified (Table 3). CGN16275 and CGN16277 had significantly greater

Table 2 Variation for big-vein symptoms in 10 *L. virosa* accessions and the *L. sativa* cultivars Pavane and Great Lakes 65 under greenhouse conditions

Accession or cultivar	Big-vein symptom incidence		MLBVV incidence	
	No. plants tested	Percent symptomatic	No. plants tested	Percent positive[a]
CGN16272	34	9	9	89
CGN16273	35	17	12	92
CGN16274	32	13	10	100
CGN16275	34	15	NT	
CGN16276	34	6	NT	
CGN16277	35	3	NT	
SAL177	34	3	12	33
Pavane	35	9	NT	
IVT280	34	0	12	17
PI271938	34	0	NT	
SAL012	23	0	12	0
Total	364	21	67	49
χ^2		19.5*		
Great Lakes 65	45	89	6	100

[a] Based on nucleic acid hybridization, expected values were to small to perform a conclusive chi square test with MLBVV incidence data

NT, not tested

* $P < 0.05$ with 10 df

Table 3 Variation for big-vein symptom expression and *Mirafiori lettuce big vein virus* (MLBVV) incidence among seven *L. virosa* accessions tested in a growth chamber experiment and evaluated at 120 and 180 days after planting (DAP)

Accession	120 Day after planting			180 Day after planting		
	Number of plants tested	Percent big-vein symptomatic[a]	Percent MLBVV positive[b]	Number of plants tested	Percent big-vein symptomatic[a]	Percent MLBVV positive[b]
CGN16275	11	45 b	73 b	10	82 a	80 c
CGN16276	26	0 a	8 a	26	31 b	77 c
CGN16277	11	27 b	55 b	11	82 a	91 c
SAL195	43	0 a	24 a	9	0 b	29 bc
IVT280	58	0 a	24 a	50	0 b	51 bc
PI274378	59	0 a	10 a	50	0 b	8 ab
SAL012	60	0 a	8 a	56	0 b	0 a

[a] Percentages with different letters are significantly different at $P < 0.05$ based on analysis of arcsine transformed values

[b] Based on nucleic acid hybridization

percentages of symptomatic plants than the remaining accessions at 120 DAP and 180 DAP, respectively. Symptomatic plants were not observed in CGN16276 at 120 DAP, but 31% of plants developed symptoms by 180 DAP. No plants with vein banding symptoms were observed in accessions SAL195, IVT280, PI274378, and SAL012 at 120 DAP or 180 DAP. Variation for the percentage of MLBVV positive plants was found at both testing dates. At 120 DAP, all accessions had MLBVV positive plants and the percentage of positive plants ranged from 8% (SAL012 and CGN16276) to 73% (CGN16725). The percentage of MLBVV positive plants in accessions CGN16275 and CGN16277 was significantly higher than the remaining accessions. By 180 DAP, the percentage of MLBVV positive plants had increased in accession CGN16275, CGN16276, CGN16277, SAL195, and IVT280 while the percentage of MLBVV positive plants decreased in PI274378 and SAL012. This resulted in SAL012 having a significantly lower percentage of MLBVV positive plants than every accession except PI274378, and with PI274378 having a significantly lower percentage of MLBVV positive plants than CGN16275, CGN16276, and CGN16277. IVT280, which remained

symptomless throughout each experiment, had 51% MLBVV positive plants by 180 DAP.

Discussion

Lactuca virosa has genetic variation for symptom expression. Furthermore, complete resistance to big-vein disease, the consistent and complete absence of symptoms, appears to be wide spread in *L. virosa*. This is a significant finding since extensive screening of diverse *L. sativa* accessions has only discovered partial resistance, and complete resistance has not been found in *L. sativa* (Hayes and Ryder, unpublished). *L. virosa* is generally considered to be a biennial or slow bolting species, although annual accessions are known. An association between resistance and slow plant development would limit the usefulness of resistance in *L. virosa*. However, big-vein symptom expression in the accessions we have tested appears to be independent of this characteristic. Vein clearing was observed in the biennial/slow bolting accessions SAL177, CGN16272, CGN16273, CGN16274, CGN16275, CGN16276, and CGN16277. Furthermore, the complete lack of vein clearing symptoms was observed in an annual accession (SAL195) and numerous biennial accessions.

Symptomless MLBVV infections in susceptible and partially resistant cultivars of lettuce have been widely reported (Hayes et al. 2006; Navarro et al. 2004; Roggero et al. 2003). We have shown in this research that *L. virosa* may also have symptomless infections of MLBVV. It is clear that virus accumulation in *L. virosa* can be a slow process, and previous studies may not have been allowed to continue long enough to detect MLBVV accumulation in *L. virosa*. In this research, extending the length of the experiments to 180 DAP was likely an important factor in discovering MLBVV symptomless infection. Furthermore, it also seems likely that the "faint" NASH results observed with some accessions in the 2004 experiment were MLBVV positive plants that had not had sufficient time to accumulate detectable quantities of MLBVV. Introgression of big-vein resistance from IVT280 into *L. sativa* was not successful in identifying hybrid lines with complete resistance (Hayes and Ryder 2007). The discovery that IVT280 can have high percentages of MLBVV positive plants despite being asymptomatic may explain the failure of this breeding effort. Additional breeding should be conducted with *L. virosa* accessions that have low percentages of MLBVV positive plants, such as accessions SAL012 and PI274378. Big-vein symptom expression is environmentally dependent (Walsh 2004). Therefore, it is not known how these *L. virosa* accessions will perform beyond 180 DAP or in environments that are further conducive to big-vein symptom expression. Importantly, most lettuce crops require only 60 to 90 days from planting to harvest, and if measurable accumulation can be delayed in *L. virosa-L. sativa* hybrids until after 100 days, this should substantially reduce the potential for big-vein disease development.

Genetic variation exists among *L. virosa* accessions for the incidence of MLBVV infected plants, and includes accessions with low percentages of MLBVV positive plants. This is most likely due to direct resistance to the virus, although the role of resistance to *O. brassicae* has not been investigated. Taken together, three categories of *L. virosa* accessions can be considered. These categories are: (1) accessions that are symptomatic with high percentages of MLBVV positive plants, (2) accessions that are completely and consistently asymptomatic despite having a large number of plants with detectable concentrations of MLBVV, and (3) accessions that are completely asymptomatic with a low incidence of MLBVV. The finding of variation for MLBVV accumulation within accessions that are completely asymptomatic suggests that symptom expression and MLBVV accumulation may be independent factors in *L. virosa* contributing to big-vein resistance. Additional research to determine the rate of MLBVV accumulation in *L. virosa* and *L. virosa-L. sativa* hybrids is needed to further characterize these accessions and to support the introgression of lettuce big-vein resistance from *Lactuca virosa* into cultivated lettuce.

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